Effect of Topical Retinoic Acids on the Levels of Collagen mRNA During the Repair of UVB-Induced Dermal Damage in the Hairless Mouse and the Possible Role of TGF-β as a Mediator

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Topically applied retinoic acids have been found to enhance the gene expression for collagen types I and III in the skin of UVB-irradiated hairless mice. Prior damage is required because the effect is not observed in the skin of age-matched, non-irradiated control animals. Immunochemical methods have shown an increase in TGF-β1 and, to a lesser extent, of TGF-β2 in the epidermis following retinoic acid treatment. There were no changes in mRNA levels for any of the isotypes of TGF-β induced by retinoic acid treatment. This study suggests that TGF-β may mediate the effect of retinoic acids on dermal repair through the stimulation of collagen gene expression. *J Invest Dermatol 98:359–363, 1992*

Numerous studies on the hairless mouse model of photodamage have demonstrated alterations in collagen after chronic UVB irradiation, either by histologic criteria [1,2] or, more recently, by biochemical techniques [3–5] or radioimmunoassay [6]. Histochemical changes have been interpreted as loss of staining due to "damage" [1,2,7] and in one study this was apparently linearly related to the cumulative UVB dose. However, change in collagen content was only observed at the longest exposure time and this change was only evident when expressed per unit tissue weight and not per unit area [8]. A recent study demonstrated a substantial loss of soluble type III procollagen without a significant alteration in the percentage of this form relative to type I [6]. Spontaneous repair of damaged collagen can take place when the irradiation is discontinued [1,2], with the appearance of a normalized dermis; retinoic acid was shown to accelerate this process [9]. A recent report [10] showed that both all-*trans*- and 13-*cis*-retinoic acids were active in a dose-dependent manner in inducing a repair zone and in effacing the UVB-induced wrinkling.

Transforming growth factor β (TGF-β) is a potent regulator of the growth and differentiation of most cells and of extracellular matrix (ECM) deposition [11,12]. Although TGF-β stimulates both fibronectin and collagen mRNA and protein synthesis and secretion, it also prevents their loss by inhibiting the enzymes involved in the breakdown of the ECM, such as plasminogen activators and collagenase [13,14]. Accordingly, in vivo studies have shown that TGF-β stimulates wound repair by its growth factor effect, by enhanced production of ECM proteins, and by its chemotactic activity [15].

The present study describes the effect of topical retinoic acids on the levels of collagen mRNA during the repair of UVB-induced dermal damage and the possible role of TGF-β as a mediator of the effect. A preliminary report of some of these data has been presented [16].

MATERIALS AND METHODS

Animal-Treatment Schedules Hairless mice (female, HRS/J strain, Jackson Labs, 5–7 weeks old at the start of the experiments) were housed in yellow light and irradiated three times per week with a bank of eight Westinghouse Sunlamps (FS40) placed about 20 cm above the animals. The radiation dose was controlled by an International Light Model IL844A Phototherapy Exposure Control and a model SEE240 detector. The UVB dosing schedule was such that individual doses, seldom exceeding 0.06 J/cm^2, caused minimal erythema but no burning or scarring. There was significant elastosis, detected by histology, after a total dose of 3.83 J/cm^2 (accumulated over a period of 22 weeks). Age-matched animals were used as non-irradiated controls.

Retinoid Treatment To effect repair of the UVB-induced dermal damage, the irradiation was discontinued and the animals were divided into groups of eight and treated three times per week with either 25 μg (0.082 μmole, 100 μl of 0.025% solution) of all-*trans*-retinoic acid (tretinoin) or 100 μg (0.33 μmole, 100 μl of 0.1% solution) of 13-*cis*-retinoic acid (isotretinoin) dissolved in acetone. These doses of retinoic acids have been shown to give optimum degrees of dermal repair. This dose of all-*trans*-retinoic acid was also the highest non-lethal dose that could be administered. Stock solutions were made up fresh every week in subdued light; dosing solutions were prepared by dilution so that the dose was delivered in 100 μl volume applied topically with a plastic pipette to an area of about 10 cm^2 on the back of the animal. All dosing was done under yellow light. A control group was treated with acetone alone. Between applications, solutions were stored under argon at −70°C. At various times of treatment animals were killed and skin samples taken for RNA isolation.

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Abbreviations:
GAPDH: glyceraldehyde phosphate dehydrogenase
KLH: keyhole limpet hemocyanin
TFA: trifluoroacetic acid
TGF-β: transforming growth factor beta
UVB: ultraviolet B (290–320 nm)

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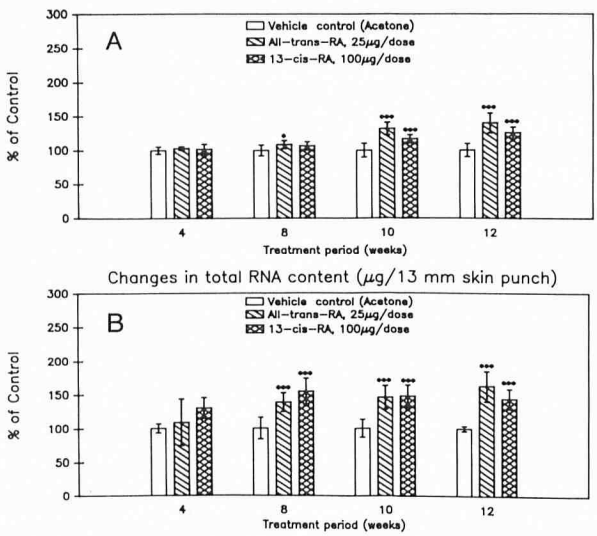

Figure 1. Tissue weights *(A)* and total RNA content *(B)* throughout the course of treatment of UVB-irradiated hairless mice with retinoic acids. $^{*}p < 0.05$, $^{***}p < 0.001$ versus control.

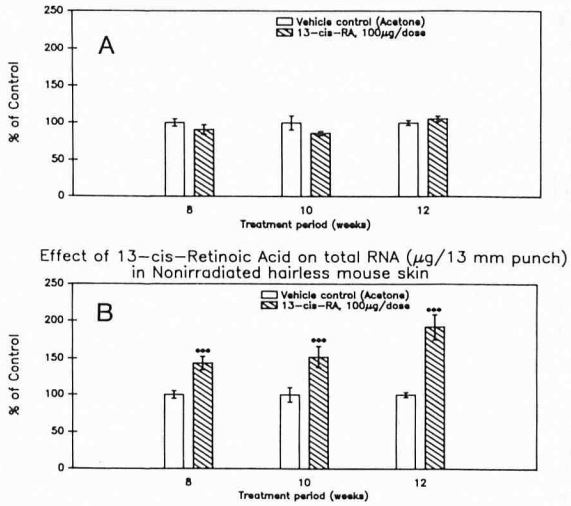

Figure 3. Tissue weights *(A)* and total RNA content *(B)* throughout the course of treatment of nonirradiated hairless mice with retinoic acids. $^{***}p < 0.001$ versus control.

RNA Isolation RNA was isolated by the guanidinium thiocyanate-phenol-chloroform procedure [17]. Reagents were the best available quality (Gibco BRL). Animals were sacrificed by cervical dislocation and skin samples (two to four 13-mm punches) were taken and rapidly homogenized, using a Polytron, in 3 ml of 4 M guanidinium thiocyanate containing 0.025 M sodium citrate, pH 7, 0.5% sarcosyl and 0.1 M mercaptoethanol. Precautions were taken to prevent contamination by ribonucleases; samples were shown to be essentially intact by agarose gel electrophoresis.

Slot Blot Hybridization Analysis Varying amounts of total RNA (0.2, 1, and 5 μg per slot) were blotted onto nitrocellulose membranes and probed with ^{32}P-labeled α_1(I)- or α_3(III)-collagen cDNA, Hf-677 [18] and Hf-934 [19], respectively. For a comparison of the retinoic acid effect on other ubiquitously expressed genes in skin tissue, a parallel membrane was probed with a ^{32}P-labeled β-actin cDNA. In the cases when the levels of collagen mRNA were assayed per unit area of skin, the blots of the same aliquots from each RNA sample preparation were assayed and corrected by the recovery index of specific mRNA during the isolation procedure. For the estimate of recovery index, 100 ng of purified rabbit β-globin mRNA was added to each tissue homogenate at the beginning of the isolation as an internal standard and the amount determined in the final preparations after a parallel membrane was probed with human β-globin cDNA [20]. The [^{32}P]cDNA-mRNA hybridization products were subjected to autoradiography by exposing the membranes to X-ray film and quantitated by scanning the densitograms at 700 nm with an automatic densitometer (Bio-Rad model 620). The relative levels of specific mRNA were evaluated based on the area under the absorbance peaks. Densitograms yielding linear regressions through three points were used as a measure for the value of mRNA levels.

Northern Blot Analysis for TGF-β mRNA A northern blot membrane of total RNA, 30 μg per sample, was subjected to serial

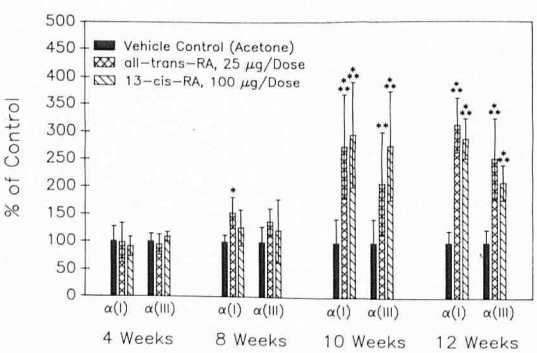

Figure 2. Steady-state levels of mRNA for type I and type III collagen during the course of treatment with retinoic acids. Values are corrected for total RNA and for β-actin mRNA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control.

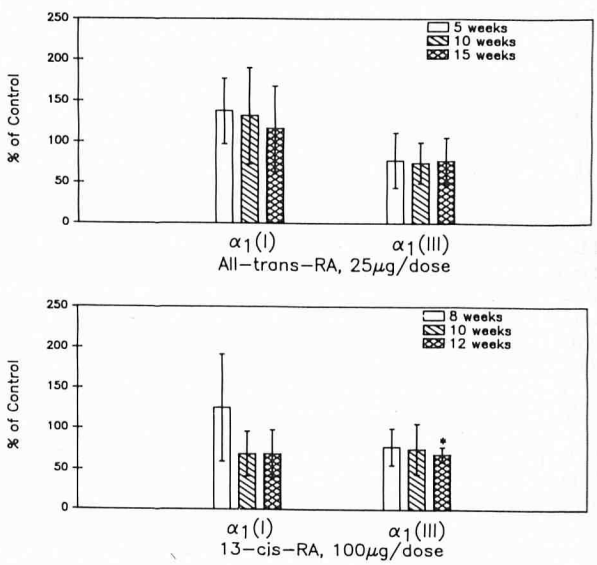

Figure 4. Steady-state levels of mRNA for type I and type III collagen during the treatment of nonirradiated hairless mice with retinoic acids. $^{*}p < 0.05$ versus control.

Table I. Effect of 13-cis-Retinoic Acid on Collagen Gene Expression in UVB-Irradiated Hairless Mouse Skin[a]

Treatment Groups		Tissue weight (mg/13 mm punch, percent of control)	Yield of RNA (μg/13 mm punch, percent of control)	Level of Collagen mRNA	
				α_1(I)	α_1(III)
A					
Control (10 weeks)	n = 5	100.0 ± 9.34	100.0 ± 7.90	100.0 ± 35.85	100.0 ± 22.46
1 week + 9 weeks	n = 5	84.1 ± 7.20	102.1 ± 3.46	184.9 ± 98.64	117.9 ± 41.77
2 weeks + 8 weeks	n = 4	88.8 ± 11.50	102.5 ± 6.33	174.6 ± 86.55	141.7 ± 35.34
3 weeks + 7 weeks	n = 4	95.9 ± 14.01	99.1 ± 5.83	204.5 ± 112.87	119.2 ± 40.26
5 days + 9 weeks	n = 3	91.8 ± 21.22	89.0 ± 14.57	168.3 ± 34.55	117.9 ± 19.43
10 days + 8 weeks	n = 5	97.8 ± 6.95	110.0 ± 9.21	214.8 ± 105.63	113.7 ± 55.86
B					
Control (10 weeks)	n = 6	100.0 ± 13.59	100.0 ± 11.17	100.0 ± 45.19	100.0 ± 40.41
6 weeks + 4 weeks	n = 6	119.1 ± 17.31	107,7 ± 8.46	110.3 ± 22.00	130.6 ± 43.39
8 weeks + 2 weeks	n = 6	110.9 ± 6.58	112.7 ± 11.29	95.5 ± 11.02	114.4 ± 52.32
Control (12 weeks)	n = 5	100.0 ± 8.39	100.0 ± 5.92	100.0 ± 34.21	100.0 ± 34.62
6 weeks + 6 weeks	n = 5	116.1 ± 4.69	105.4 ± 12.80	100.0 ± 50.70	87.6 ± 13.87
8 weeks + 4 weeks	n = 4	110.0 ± 20.24	115.6 ± 12.41	114.9 ± 33.23	109.1 ± 20.31

[a] The total experimental period was either 10 or 12 weeks, consisting of a treatment phase followed by the balance of the time during which the animals were not treated. Other groups were left untreated for various times before treatment started and continued for the balance of the experiment. *A*: animals were treated for various times with 13-*cis*-retinoic acid then left untreated for the balance of the 10-week study period. *B*: animals were untreated for various time periods then treated with 13-*cis*-retinoic acid for the balance of the study period (either 10 or 12 weeks).

hybridization with hTGF-β1, mTGF-β2, TGF-β3, and GAPDH cDNA.

A plasmid containing a 1.6-kb human TGF-β1 cDNA was obtained from Dr. M. Ostrowski, Duke University. A 1.05-kb Bgl/1 fragment of the cDNA was used to probe for TGF-β1-specific mRNA in mouse samples. Plasmids infected with mouse TGF-β2 and TGF-β3 were obtained from Dr. M. Sporn, NIH; 1.2-kb cDNA inserts were used to assay the levels of gene expression.

Preparation of Antisera Peptides of TGF-β1 and TGF-β2, residues 4–19, and TGF-β3, residues 9–20, were synthesized using a 430A peptide synthesizer (Applied Biosystems, Inc.). A detailed description of the preparation and characterization of the antisera is reported elsewhere [21]. Briefly, the peptides were purified by HPLC and 5 mg of each coupled to KLH at a 1 : 1 ratio (w/w), using 1.25% glutaraldehyde. Rabbits were immunized with 500 μg of each peptide initially, subsequently boosted with 250 μg and the antibody titer determined by ELISA using appropriate corresponding uncoupled peptide. The antisera did not indicate cross-immunoreactivity with peptides not used as immunogen and each antiserum was purified by ammonium sulfate precipitation (31.3%), followed by peptide affinity chromatography using the respective immunogenic peptide. Each anti-peptide antiserum was tested for cross-reactivity with each other TGF-β isoform by Western blot analysis [21].

Immunostaining Immunostaining was performed using an immunoperoxidase system (Vectastain Elite ABC Kit from Vector Laboratories, Burlingame, CA). Paraffin sections were reacted with appropriate dilutions of the antisera and the remaining operations carried out according to the manufacturer's instructions.

RESULTS

Figure 1 shows the tissue weights and total RNA content of the tissue throughout the course of retinoic acid treatment, expressed relative to controls. Vehicle-treated control decreased slightly during the time of treatment. Treated tissue weights increased and the differences were statistically significant at 10 and 12 weeks. RNA content increased, reflecting an increase in the cellularity of the tissue. The levels of collagen mRNA are shown in Fig 2. No increments are observed for up to 8 weeks but levels of both types I and III increase two to three fold at 10 and 12 weeks and are statistically significant. The levels of collagen mRNA in total RNA were corrected for recovery during extraction by a factor as described in *Materials and Methods* and are normalized to levels of β-actin. Thus they represent specific stimulation of collagen gene expression.

To test for the requirement for prior UVB damage on the retinoid effect, age-matched, non-irradiated animals were treated with retinoic acids and the tissues examined at various time intervals. Figure 3 shows that the tissue weight changes were not significant but that there were substantial increases in RNA content from 8 weeks onwards. Tissue weights in the control group showed no significant changes. The levels of mRNA for types I and III collagen did not change significantly during this time (Fig 4), indicating that prior tissue damage is required for a retinoic acid response.

Continual presence of the isotretinoin is required for the effect. When animals were dosed for 1, 2, or 3 weeks and then untreated for the balance of the 10-week period no significant increments in specific collagen gene expression were observed (Table 1*A*). We have no explanation for the relatively high values, in some cases, for α_1(I) collagen mRNA or for the large standard errors. The same result was obtained for animals treated with 5 or 10 consecutive daily doses of isotretinoin. To test for the possibility of a refractory period during which the tissue does not respond to isotretinoin, animals were untreated for 6 or 8 weeks following cessation of the UVB irradiation, then treated with isotretinoin for the balance of a total period of 10 or 12 weeks. As can be seen in Table 1*B*, no effect on collagen gene expression was evident. Other data (not shown) confirm that in the same experiment continuous treatment for 10 to 12 weeks gave statistically significant increments in the levels of collagen mRNA similar to those reported in Fig 2.

Because TGF-β has been shown to induce collagen synthesis we examined the pattern of immunostaining with antiserum to TGF-β1 on sections of skin treated for 10 weeks with either vehicle (Fig 5*B*) or 13-*cis*-retinoic acid (Fig 5*A*). Figure 5 illustrates that significant immunoreactivity is evident in the upper layers of the epidermis, sebaceous glands, and hair follicles following UVB irradiation and is then further enhanced severalfold after treatment with 13-*cis*-retinoic acid. Less staining is found in the dermis. Essentially similar results were obtained for the antibody to TGF-β2. The antibody to TGF-β3 was not immunoreactive.

Examination of tissues taken at earlier time points in the repair protocol suggested that 13-*cis*-retinoic acid treatment had little enhancing effect on the immunostaining at the 2-, 4-, and 6-week time points.

Northern analysis of total RNA demonstrated abundant levels of transcripts for TGF-β1, TGF-β2, and TGF-β3 but there were no differences between control and tissue treated for 10 weeks with 13-*cis*-retinoic acid (Fig 6).

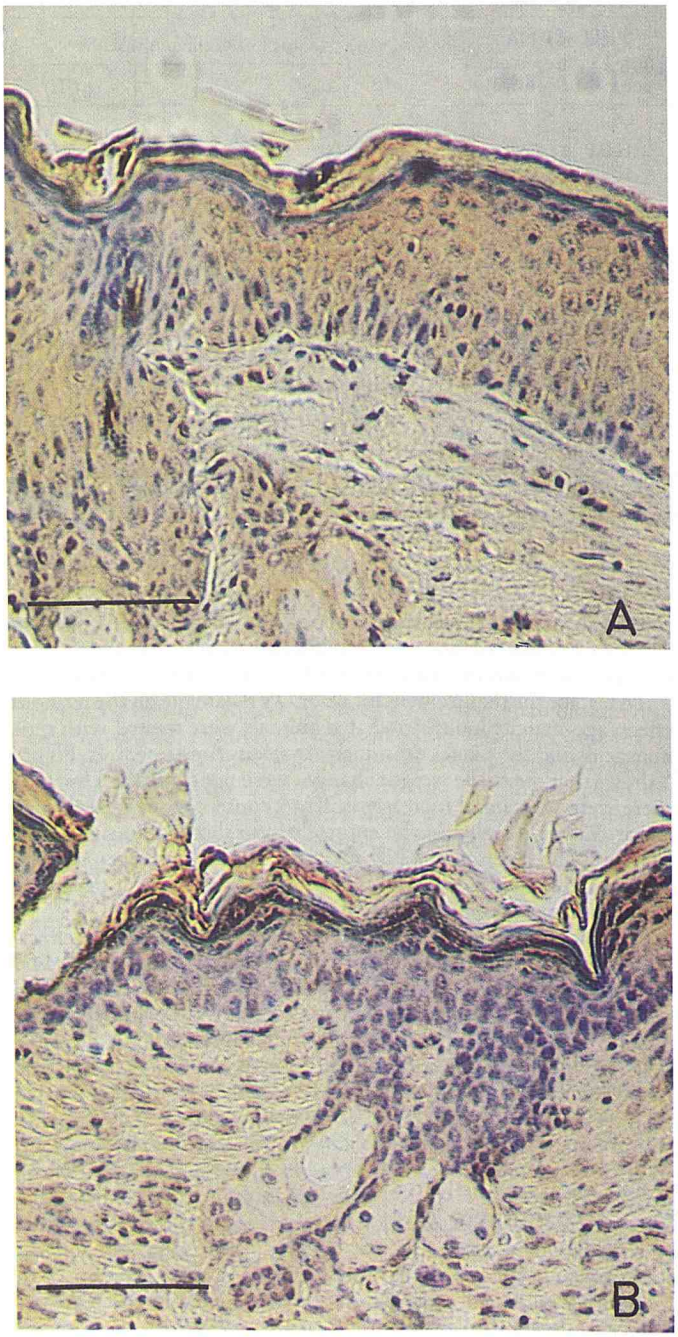

Figure 5. Immunostaining for TGF-β1. *A*) tissue treated for 10 weeks with 13-*cis*-retinoic acid and *B*) tissue treated with vehicle (acetone). *Bar*, 100 μm.

DISCUSSION

Numerous studies of skin fibroblasts in culture have shown an effect of retinoic acids. Growth of adult human skin fibroblasts was suppressed by 10^{-5} M 13-*cis*-retinoic acid [22]. At low retinoid concentrations (10^{-9} M), general protein synthesis and collagen production were equally inhibited; at higher concentrations (10^{-5} M) collagen production was slightly more affected than non-collagenous protein synthesis. Both types I and III were equally reduced. More recent studies using short-term incubations in serum-free conditions [23] demonstrated selective inhibition of collagen synthesis by 13-*cis*-retinoic acid and all-*trans*-retinoic acid (at 10^{-5} M), which was accompanied by a similar reduction in the level of proα2(I) mRNA for type I procollagen. Control experiments showed that incubation with the retinoids did not affect post-translational hydroxylation of prolyl residues of collagen, the specific radioactivity of the intracellular prolyl transfer RNA pool, or DNA replication. Interestingly, the production of collagenase was simultaneously reduced.

In view of the apparently contradictory results, the relevance of studies with cultured fibroblasts in the in vivo situation is not clear at present. Retinoids are known to give biphasic responses with cultured cells and growth conditions can alter the final result. A recent study found that human skin fibroblasts, neonatal and adult, responded to retinoic acid when growth had been arrested by culture in serum-free medium containing only 0.15 mM calcium [24]. The growth stimulation occurred within a narrow range of retinoid concentrations (1.7 to 6.6 μM). More significant was the observation that the same concentrations of retinoid caused a stimulation of synthesis of extracellular matrix components, collagen, laminin, and thrombospondin. These changes mimic those seen in the dermis when photodamaged skin is treated with retinoic acids.

The time-course of the effect is puzzling. Dermal repair appears to proceed linearly with time ([9], G.F. Bryce, unpublished observations) although it is difficult to detect at early times (during the first few weeks) but it is clearly evident at 5 to 6 weeks, at which time the collagen mRNA levels have not yet increased. Two recent studies have reported a similar lag phase. Using antibodies against the aminopropeptide of type III collagen, Kligman et al [25] observed that, in UVB-damaged tissue, immunoreactivity was evident throughout the entire dermis in control animals and in animals treated with 0.05% all-*trans*-retinoic acid after 2, 4, and 6 weeks but increased markedly in a subepidermal zone at 8 and 10 weeks in the treated animals. In an experiment with essentially the same protocol, Chen et al [26] measured collagen synthesis during the all-*trans*-retinoic acid treatment of UVB-irradiated hairless mice. No effect was evident until 6 weeks into the treatment at which point the stimulation was 165% of control values; this value increased to

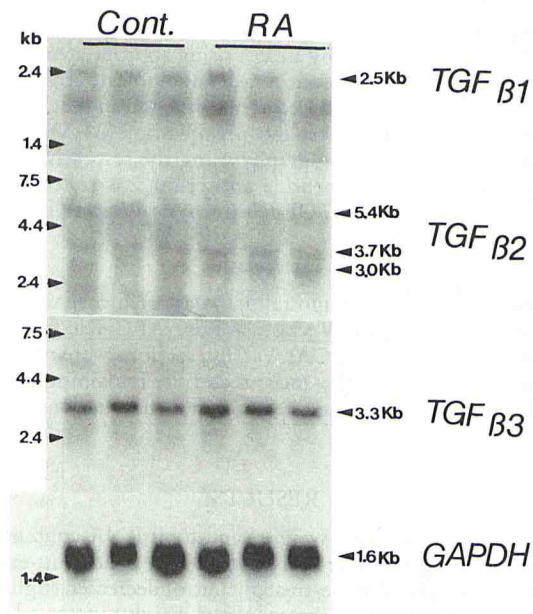

Figure 6. Northern analysis of RNA isolated from UVB-irradiated mouse skin treated for 10 weeks with 13-*cis*-retinoic acid (RA) and probed for TGF-β1, TFG-β2, TGF-β3, and GAPDH. *Arrows* and figures on the left hand side denote the positions of molecular weight markers. Transcript sizes agreed with published values: 2.5 kb for TGF-β1; 3.0, 3.7, and 5.4 kb for TGF-β2, and 3.7 kb for TGF-β3.

245% at 10 weeks. As in the studies presented above, no effect was observed on animals not previously exposed to UVB.

The possibility of an indirect effect of retinoids is suggested by recent data implicating TGF-β. This factor is one of a small number of molecules that promote the synthesis of collagen and it is known to act by increasing transcription of mRNA for several connective tissue collagens [13,27]. Retinoic acid stimulates the production of both TGF-β2 protein and mRNA in mouse keratinocytes [28] and was recently shown by immunohistochemical methods to stimulate production of TGF-β1 protein but not mRNA in human skin [29]. The studies reported here show for the first time a retinoic acid–stimulated increase in immunostaining for TGF-β1 and, to a lesser extent, TGF-β2 in the HRS/J mouse without any concomitant changes in the levels of mRNA. A possible explanation for this apparent discrepancy is that the retinoic acid stimulates processing of pre-formed TGF-β protein to a form recognized by the antibodies used in this study. The time course of TGF-β1 immunostaining appears to parallel the collagen mRNA profiles in that a lag phase is present up to 6 to 8 weeks following initiation of treatment. Stimulation of the TGF-β1 form described here is in contrast to the results of Glick et al [28] where the predominant form was reported to be TGF-β2. The reason for the discrepancy is not apparent at present, although different antisera and mouse species were used.

Thus a plausible mechanism for isotretinoin action in the photodamage repair model would be an initial stimulation of epidermal cells to produce TGF-β, which subsequently stimulates dermal fibroblasts to synthesize collagen.

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